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13. ABSTRACT *(Maximum 200 words)*

The goal of the proposed research is to develop breast tumor-avid peptides as potential in vivo imaging or therapeutic agents. Peptides that specifically bound two breast tumor antigens, the Thomsen-Friedenreich (T) antigen and the erb-B2 receptor, were identified from random peptide bacteriophage display libraries. The T antigen-avid peptides were labeled with ^{99m}Tc and shown to be radiochemically stable and bind cultured human breast tumor cells in vitro. In vivo biodistribution studies performed in normal mice showed that the peptides were stable in vivo and exhibited whole body clearance primarily through the GI tract. Tumor targeting studies with the ^{99m}Tc-labeled peptides will begin shortly in breast tumor bearing mice. In addition, bacteriophage display libraries, displaying random peptides of various lengths, were screened with recombinant erbB-2 extracellular domain protein. Sequencing of erbB-2 binding clones has yielded the first consensus peptide. More clones, will be sequenced and analyzed to identify additional erbB-2 binders. The erbB-2 binding peptides will be chemically synthesized, radiolabeled, and examined for their abilities to bind breast tumor cells in vitro and in vivo.

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INTRODUCTION:

The focus of our DoD funded research program is to identify peptides that specifically bind two breast tumor antigens, the Thomsen-Friedenreich (T) antigen and the erb-B2 receptor, radiolabel the peptides with ^{99m}Tc and ^{188}Re, and determine the abilities of the radiolabeled peptides to target breast tumors in a scid mouse model system. The goal of this work is to develop breast tumor-avid peptides as potential in vivo imaging or therapeutic agents.

Background of previous work: T antigen is a disaccharide (Galß1 → 3GalNAc) which is masked on the surface of healthy cells, but is exposed and immuno-reactive on the surfaces of most tumor cells (1), particularly breast carcinoma cells (2). Peptides that bound T antigen were identified from a random peptide phage display library, screened with two glycoproteins which display T antigen on their surfaces, asialofetuin and a BSA-T conjugate. Eight-hundred affinity selected clones were sequenced. The most frequently occurring phage clones, P10, had the sequence; Gly-Ser-Trp-Tyr-Ala-Trp-Ser-Pro-Leu-Val-Pro-Ser-Ala-Gln-Ile. P10 and next most frequent sequence P30 (His-Gly-Arg-Phe-Ile-Leu-Pro-Trp-Trp-Tyr-Ala-Phe-Ser-Pro-Ser) were chemically synthesized. Fluorescence quenching titration assays were used to measure the dissociation constants (K_ds) for P10 and P30 binding to T antigen present on asialofetuin. P10 and P30 exhibited K_ds of 100 nM and 0.1 μM, respectively, for binding the T antigen structure *in vitro*. The ability of P10 and P30 to interact with the native conformation of T antigen presented on tumor cells was confirmed by visualization of biotinylated-P10 and -P30 bound to MDA-MB435 human breast carcinoma cells. Biotin was attached to the amino termini of the peptides. Biotinylated-P10 and -P30 were detected by the binding of a streptavidin-horseradish peroxidase conjugate and subsequent catalysis of the chromogenic substrate DAB. Biotinylated-P10 and -P30 bound to the MDA-MB435 human breast carcinoma cells, and were competed off by the presence of excess non-biotinylated peptide. The presence of T antigen on the MDA-MB435 cells was confirmed by binding of a peroxidase-peanut lectin conjugate. Murine melanoma B16-F1 cells, which do not display T antigen, were used as a negative control. These results were published in the *Journal of Molecular Biology* (3). Our goal was to radiolabel P10 and P30 and examine their potential as breast tumor imaging agents.

BODY:

Results:

A. T- Antigen Project:

1. *Radiolabeling T antigen peptides:*

Analogs of the T antigen binding peptides P10 and P30 were synthesized with either an acetylated-Cys-Gly-Cys-Gly (Ac-CGCG) or Cys-Gly-Cys-Gly (CGCG) tetrapeptide appended to their amino termini (Table 1)

Table 1. Peptide Synthesized

Name	Peptide Sequence
Ac-CGCG-P30	Ac-CGCG-HGRFILPW**WYAFSP**S
Ac-CGCG-P54	Ac-CGCG-WHR**WYAWSP**
CGCG-P54	CGCG-WHR**WYAWSP**
Ac-CGCG-P55	Ac-CGCG-R**WYAWSP**
CGCG-P55	CGCG-R**WYAWSP**
Ac-CGCG-P10	Ac-CGCG-GS**WYAWSP**LVPSAQI

* Putative T antigen binding sequences are highlighted in bold type.

We had previously shown that the CGCG peptide was able to coordinate $^{186/188}$Re and ^{99m}Tc when it was attached to the N-terminus of polypeptide (4). The peptides in Table 1 were synthesized on an ABI-432 amino acid synthesizer using standard Fmoc solid phase peptide synthesis (SPPS) chemistry (4). Peptides were cleaved from the resin with a 95:5 trifluoroacetic acid (TFA):water

mixture and deprotected in a 50:10:40 TFA:thioanisol:water mixture, ether precipitated, and lyophilized. Peptide preparations were purified to homogeneity on a Vydac C-18 reverse-phase (RP) column using high performance liquid chromatography (HPLC). The masses of the purified peptides were determined by fast-atom bombardment mass spectroscopy (FAB-MS). The predicted masses of Ac-CGCG-P10 and -P30 agreed with the experimentally determined values.

The T antigen binding properties of Ac-CGCG-P10 and -P30 were examined in vitro by fluorescence quenching experiments as previously described in the background section. It was important to demonstrate that the addition of the N-terminal radiometal coordination tetrapeptide did not effect T antigen binding. The K_d values for the Ac-CGCG-P10 and -P30 peptides were the same as the values determined for P10 and P30, within experimental error, indicating that the additional amino acids did not effect T antigen binding. Ac-CGCG- and CGCG- containing peptides were radiolabeled with ^{99m}Tc directly via a glucoheptonate transchelation reaction (4, 5). Briefly, ^{99m}TcO VII was reduced to ^{99m}TcO V by stannous chloride in the presence of glucoheptoic acid, forming a ^{99m}TcO V-glucoheptonate complex. Peptide was added to the reaction mixture and the pH of the solution was adjusted to 7.0 with NaOH. The reaction mixture was refluxed at 80°C for 40 min. Under these conditions, the Cys sulfurs of the CGCG sequence form a more favorable ^{99m}TcO V coordination complex than glucoheptonate, which drives the peptide radiolabeling reaction. The stabilities of ^{99m}Tc labeled peptides were examined after a 24 hr incubation in phosphate buffered saline, pH 7.4 (PBS) and in PBS containing 4 mM cysteine (Table 2).

Table 2 Stability of Radiolabeled Peptides in PBS and PBS+ Cysteine 24 hr post purification.

Tc-99m-Radiolabeled Peptides	In PBS(10mM)	Cysteine(4mM)
Tc-99m-Ac-CGCG-P30	N.D.	N.D.
Tc-99m-Ac-CGCG-P10	N.D.	N.D.
Tc-99m-Ac-CGCG-P54	N.D.	40% remain
Tc-99m-CGCG-P54	N.D.	90% remain
Tc-99m-Ac-CGCG-P55	N.D.	30% remain
Tc-99m-CGCG-P55	N.D.	40% remain

*N. D.= No degradation

The radiolabeled peptide were injected on a C-18 RP column which is capable of separating labeled peptide from pertechnetate (free ^{99m}Tc). The results indicated that the ^{99m}Tc labeled peptides were stable in vitro over a 24 hr time period. In addition, the radiolabeled peptide were resistant to chemical challenges from free cysteine

2. *Cell Binding Experiments*:

Cell binding experiments were performed with cultured human breast cancer MDA-MB-435 cells (Table 3). T antigen expression on MDA-MB-435 cells was confirmed by a positive binding reaction with peanut lectin (3).

Table 3. Cell binding to MB435 for technetium labeled peptides 4°C for 1.5hr

Peptide	TB[1]	NSB[2]	SB[3]
Ac-CGCG-P30	27.7%	11.0%	16.7%
Ac-CGCG-P54	4.65%	2.23%	2.42%
Ac-CGCG-P55	1.12%	0.75%	0.37%
Ac-CGCG-P10	5.12%	4.54%	0.58%

[1]TB, total binding; [2]NSB, non-specific binding; [3]SB, specific binding.

Briefly, $2x10^6$ cells were mixed with approximately 100,000 cpm of a radiolabeled peptide. The mixture was allowed to incubate at 4°C for 2 hrs. The cells were washed 2x with PBS and counted to determine total binding. Non-specific binding experiments were performed in a similar fashion

except that a 1000-fold excess of unlabeled peptide was present. Specific binding was determined by subtracting the non-specific binding from total binding. The P30 and P54 peptides exhibited the highest specific binding values. In all cases, the non-specific binding was fairly high. This could be due to peptide precipitation caused by the addition of the excess cold peptide. We are repeating our binding assays in different buffers and in larger volumes to determine if peptide precipitation contributes significantly to the non-specific binding numbers.

3. *Animal Experiments*:

The biodistribution of ^{99m}Tc labeled Ac-CGCG-P30, -P54, -P55, -P10, and CGCG-P54 and -P55 were examined in C57BL/6 normal mice. Mice were injected with 3-5 μCi of a radiolabeled peptide. At various times post injection, the mice were sacrificed and specific tissues were counted. Results from the biodistribution experiments are presented in Tables 4 and 5 as percent dose per gram or as percent of injected dose, respectively.

Table 4. Biodistribution of ^{99m}Tc-peptides in C57BL/6 normal mice 1hr post-injection reported as % injected dose/gram, (n=4±SD).

Tissue	Ac-CGCG-P30	CGCG-P54	Ac-CGCG-P54	CGCG-P55	Ac-CGCG-P55	Ac-CGCG-P10
Brain	0.09±0.02	0.02±0.02	0.02±0.02	0.01±0.02	0.02±0.01	
Blood	2.24±0.43	0.38±0.07	0.59±0.05	0.13±0.07	0.90±0.04	7.24±2.73
Heart	0.55±0.07	0.09±0.06	0.24±0.08	0.12±0.12	0.26±0.06	1.38±0.72
Lung	1.35±0.26	0.22±0.04	1.10±0.20	0.15±0.02	0.57±0.20	2.16±1.26
Liver	47.04±5.28	2.96±1.56	2.07±0.55	4.59±1.75	1.43±0.40	13.86±1.26
Spleen	1.05±0.14	0.06±0.03	0.24±0.10	0.07±0.06	0.30±0.18	0.92±0.13
Stomach	3.77±3.18	0.88±0.57	2.20±0.86	0.55±0.32	6.38±1.31	2.04±0.75
Intestines	13.60±3.05	33.53±4.40	35.27±2.94	28.24±1.39	33.74±3.97	31.49±1.45
Larger I.	1.12±1.30	3.53±5.94	23.29±37.36	5.19±6.19	33.88±22.91	0.99±0.69
Small I.	21.60±4.19	48.08±8.52	41.76±18.38	40.26±2.00	33.60±15.84	48.26±2.33
Kidneys	2.42±0.23	0.83±0.12	2.41±1.18	1.75±0.23	1.18±0.04	2.53±0.14
Muscle	0.11±0.10	0.16±0.04	0.15±0.06	0.09±0.08	0.14±0.05	0.28±0.07
Pancreas	0.50±0.12	0.63±0.50	0.29±0.09	0.08±0.05	0.32±0.04	0.74±0.24

*I.;Intestines

Results from the initial biodistribution experiments indicated that the primary route of clearance for these T antigen binding peptides was through the gastrointestinal (GI) tract. All of the peptides show low uptake in the organs around the breast (i.e. lungs and heart), except for P10 which display slightly elevated activity levels in the lungs. There was good clearance from the blood and low liver uptake for P54 and P55, while the longer peptides showed higher liver values. It was no surprise that the primary route of peptide clearance was through the GI tract given the hydrophobic nature of the P10 and P30 sequences. Removing some of the non-essential hydrophobic residues of P10 and P30 resulted in the P54 and P55 sequences. It was hypothesized that increasing the hydrophilic nature of the peptides would improve their clearance. The biodistribution patterns of the P54 and P55 peptides indicated that their was less liver uptake and better blood clearance, but the primary route of clearance was still through the GI tract. These results suggested that the hydrophobic nature of the ^{99m}Tc[Ac-CGCG] complex may have a dominant impact on the peptide's clearance properties. Experiments are currently underway to investigate the role of the ^{99m}Tc[Ac-CGCG] complex in the biodistribution of our T antigen binding peptides. The presence or absence of the acetyl group on the N-terminal cysteine did not appear to have an effect on the stability of the peptides in vivo. Many peptides are rapidly proteolyzed in vivo. N-terminal acetylation affords peptides some degree of resistance to proteolysis. The fact that the N-terminal residues of the

radiolabeled T antigen peptides are involved in metal coordination may prevent them from being recognized by proteases in vivo.

Table 5. Biodistribution of ^{99m}Tc-peptides in C57BL/6 normal mice 1hr post-injection reported as % injected dose, (n=4±SD).

Tissue	Ac-CGCG-P30	CGCG-P54	Ac-CGCG-P54	CGCG-P55	Ac-CGCG-P55	Ac-CGCG-P10
Brain	0.04±0.00	0.00±0.00	0.00±0.00	0.01±0.00	0.00±0.00	
Blood	3.47±0.77	0.82±0.07	0.55±0.12	1.28±0.07	0.18±0.09	0.79±0.43
Heart	0.05±0.00	0.02±0.00	0.00±0.00	0.02±0.00	0.01±0.01	0.16±0.00
Lung	0.25±0.11	0.16±0.03	0.03±0.00	0.10±0.01	0.02±0.00	0.38±0.10
Liver	49.23±1.28	2.64±0.57	3.69±2.08	1.99±0.73	5.54±2.16	16.59±1.97
Spleen	0.07±0.01	0.02±0.01	0.00±0.00	0.02±0.01	0.01±0.01	0.07±0.01
Stomach	0.62±0.45	1.05±0.29	0.33±0.15	3.78±0.09	0.21±0.11	0.38±0.14
Intestines	25.84±1.04	70.00±1.74	75.78±3.36	73.19±1.87	65.66±1.88	58.40±3.28
Large Int.	0.94±1.21	14.77±23.5	2.52±4.21	25.00±17.4	3.93±4.66	0.58±0.39
Small Int.	24.89±0.84	55.22±23.1	73.26±4.51	48.18±18.5	61.72±4.44	51.83±3.44
Kidneys	0.69±0.01	0.65±0.04	0.25±0.04	0.36±0.03	0.54±0.04	0.76±0.06
Muscle	0.01±0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.00±0.00	0.03±0.00
Pancreas	0.13±0.02	0.05±0.01	0.14±0.10	0.06±0.01	0.01±0.01	0.17±0.02
Urine	15.02±0.54	20.99±2.55	17.28±1.16	1.26±0.69	26.55±1.15	9.68±0.43
Carcass	7.43±0.16	4.23±0.69	2.32±0.51	5.42±0.30	2.32±0.51	18.58±3.00

*I.;Intestines

B. erbB-2 Project:

1. *Expression and Purification of erbB-2:*

The erbB-2 extra cellular domain (ecd) gene, containing a C-terminal FLAG™ tag, engineered into the eukaryotic expression vector pRC/CMV (Invitrogen) was obtained from Dr. Fiddes at the CRC for Biopharmaceutical Research PTY LTD, Australia. This expression system was reported to secrete 0.5-1 μg of erbB-2ecd/ml of culture media (6). The erbB-2ecd expression plasmid, was transfected into immortalized human embryonic kidney (HEK-293) cells. The transfected cultures were placed under geneticin selection since the erbB-2 plasmid also contained a Neo resistance gene. Individual cell clones were isolated and expanded into clonal cell lines. Culture media samples from individual erbB-2 containing KB cells lines were examined for the presence of the recombinant erbB-2 protein. Cell culture media samples were concentrated 10x and used to coat enzyme-linked immunosorbant assay (ELISA) plate wells at various dilutions for 2 hr at 25°C. The wells were blocked with a bovine serum albumin (BSA)/Tween-20 solution prior to the addition of a mouse anti-erbB-2 extracellular domain antibody. An anti-mouse Fc antibody conjugated to alkaline phosphatase was employed for detection purposes. The ELISA assay was developed with the chromogenic substrate p-Nitrophenyl phosphate. This ELISA assay allowed us to determine which clone was secreting the most erbB-2 protein. The identity of the secreted protein was confirmed by Western blot analysis (Figure 1A). Aliquots from the concentrated media samples were run on reducing 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose. The Western blots were developed by the same antibody combination used in the ELISAs described above. A strong signal with an apparent molecular weight of ~90 KD was visualized. Clone # 7 was chosen for further amplification and protein production. The erbB-2 ecd protein was purified by affinity chromatography using anti-FLAG tag resin (Sigma Chemical). Briefly, 100-200 ml of culture supernatant was passed over a 5ml FLAG affinity column. The column was washed with 200 ml of TBS [20 mM Tris·HCl (pH 8.0), 150 mM NaCl]. The erbB-2ecd protein was eluted with 0.1 M glycine·HCl, pH 3.0. The pH of the erbB-

2ecd containing samples were immediately adjusted to ~8 by addition of 0.1 volume 1 M Tris·HCl, pH 8.0. The protein yields from this purification averaged 0.8 μg/ml of culture media. Samples of the protein were analyzed by reducing SDS-polyacrylamide gel electrophoresis (PAGE). As depicted in Figure 1B., a homogeneous protein species with a molecular weight of ~95 KD was present. Approximately 80 μgs of erbB-2ecd was purified for use in phage library maturation experiments.

Figure 1.

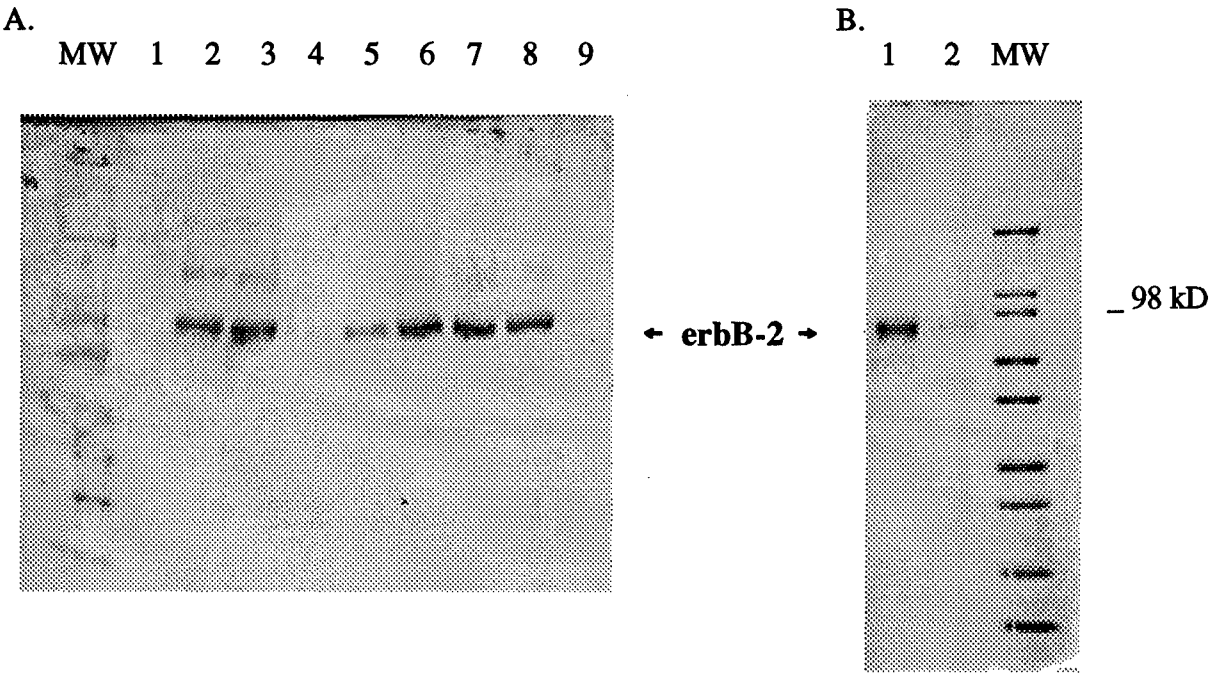

Figure 1. Analysis of erbB-2-ecd. (1A) Western blot analysis of cell culture media from cells clones expressing the erbB-2-ecd protein. (1B) SDS-PAGE analysis of erbB-2ecd after affinity purification. Lanes 1 and 2 are different concentrations of the purified erbB-2ecd preparation. Molecular weight markers (MW) are reported in kiloDaltons (kD).

2. *Affinity maturation of peptide phage display libraries with erbB-2*:
Two phage display libraries, exhibiting either 15 or 6 amino acid random peptides, were screened with the purified erbB-2ecd protein. The erbB-2ecd protein was biotinylated at a ratio of 2 biotin molecules per protein molecule in a aqueous reaction with N-hydroxsuccinimide (NHS)-biotin. Free biotin was removed from the protein preparation by G-25 size-exclusion chromatography. The affinity maturation procedure took place in 22 mm tissue culture plates. The biotinylated erbB-2ecd protein was incubated in streptavidin or neutravidin coated plates overnight at 4° C. The plates were was washed with TBS and blocked with a solution of 1% BSA in TTBS [20 mM Tris·HCl (pH 8.0), 150 mM NaCl, 0.1% Tween-20] for 2 hrs at 25°C. One plate was incubated with an anti- erbB-2ecd antibody to confirm the present of erbB-2ecd on the plates prior to performing the affinity maturation experiments. Initially, a solution containing 13 μg of erbB-2ecd was used to coat the plates. 1 x 10^{11}phage particles from either the 15 or 6 mer library were added to the erbB-2ecd coated plates in TTBS. The plates were incubated at 25°C for 2 hrs, then washed extensively with TTBS to remove unbound phage particles. Bound phage particles were eluted from the plates in 0.1 M glycine·HCl, pH 2.2. The eluted phage were used to infect a fresh culture of E. coli to amplify the selected population used in the next round a affinity maturation. Round two began with the input phage obtained from round 1 after amplification. The same selection protocol was used as

in round 1 except less biotinylated erbB-2ecd was attached to the plate. Each subsequent round of affinity maturation had less erbB-2ecd on the plate to increase the stringency of selection. In addition, neutravidin was used in round 3 and 4 instead of streptavidin to reduce non-specific binding. Table 6 lists the amount of erbB-2ecd used in each round and the input and output phage values obtained in the affinity maturation procedure.

Table 6: Affinity maturation efficiencies per round of selection.

	Round 1	Round 2	Round 3	Round 4
[biotin- erbB-2ecd] Plate coating	13 μg streptavidin	5.2 μg streptavidin	1 μg neutravidin	0.1 μg neutravidin
Inputs	6mer $1.3x10_{11}$ 15mer $1.3x10_{11}$	6mer $1.0x10_{11}$ 15mer $8.0x10_{10}$	6mer $1.2x10_{10}$ 15mer $2.8x10_{10}$	6mer $6.6x10_{9}$ 15mer $6.0x10_{9}$
Outputs	6mer $2.0x10_{4}$ 15mer $2.2x10_{4}$	6mer $2.2x10_{5}$ 15mer $2.6x10_{5}$	6mer $9.2x10_{4}$ 15mer $1.4x10_{5}$	6mer $2.7x10_{6}$ 15mer $2.4x10_{6}$
Percent yields	6mer $1.5x10\text{-}5$ 15mer $1.2x10\text{-}5$	6mer $2.2x10\text{-}4$ 15mer $3.3x10\text{-}5$	6mer $3.1x10\text{-}3$ 15mer $2.0x10\text{-}3$	6mer $4.1x10\text{-}2$ 15mer $3.9x10\text{-}2$

3. *Sequence of erbB-2 binding peptides*:

Phage colons obtained from round 4 of the affinity selection procedure were isolated and prepared for sequencing. First, clones from the 15mer library were sequenced. Thus far, the region of the CPIII gene encoding the random peptide inserts from 85 clones have been sequenced. 95% of these clones had the same peptide amino acid sequence. The sequence is; Trp-Arg-Arg-Trp-Phe-Tyr-Gln-Phe-Pro-Thr-Pro-Leu-Ala-Ala-Ala. Additional clones from the 15mer library as well as 100s of clones from the 6mer library will be sequenced to identify polypeptide sequences that bind erbB-2.

Discussion in relationship to Statement of Work:

The two tasks outlined for the first 12 months in the statement of work for this proposal were to synthesize and radiolabel T antigen peptides and identify peptide sequences that bound purified erbB-2.

Task 1: We have synthesized and radiolabeled the T antigen binding peptides P30 and P10 plus several analogs based on their conserved chemical structures. Radiochemical stabilities as well as in vitro cell binding assays have been performed with the ^{99m}Tc labeled peptides.

Task 2: (12-24 months). In vivo biodistribution and tumor targeting experiments will be performed in normal and breast tumor bearing scid mice. We have already begun in vivo biodistribution studies in normal mice to examine peptide stability and clearance patterns.

Task 3: Immortalized human cell lines expressing the extracellular domain of erbB-2 have been established. These cell lines secrete protein which is reactive with anti-erbB-2 antibodies. An affinity purification protocol has been worked out for isolating erbB-2 from the cell culture media. Purification yields for this process are 0.8 μg/ml of tissue culture media. The purified protein was used to identify peptide sequences that bound erbB-2 from random peptide phage display libraries. Initial clones from these affinity maturation procedures have been sequenced.

The remaining tasks are all scheduled for years 2 and 3.

CONCLUSIONS:

During the first 12 months of this project all of the important foundation tasks have been accomplished. We have been successful at radiolabeling our T antigen binding peptides and have shown that they are radiochemically stable. We have also been able to establish the expression of

the erbB-2-ecd protein in cultured human embryonic kidney cell lines. Affinity purification procedures have produced purified erbB-2-ecd used in screening the random peptide phage display libraries. The most important results so far are that we have begun to evaluate the ^{99m}Tc labeled T antigen peptides in vivo. The radiolabeled peptide appear to be radiochemically stable and resistant to proteolysis in vivo. Equally exciting results are being obtained from screening the random peptide phage display libraries with the erbB-2-ecd protein. A putative 15 amino acid peptide that binds erbB-2 has been identified from one of the phage libraries. This peptide will be chemically synthesized and examined for its ability to bind the erbB-2 receptor on human breast carcinoma cells.

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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